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Molecular and morphological characterization of *Contracaecum pelagicum* (Nematoda) parasitizing *Spheniscus magellanicus* (Chordata) from Brazilian waters

Caracterização molecular e morfológica de *Contracaecum pelagicum* (Nematoda) parasito de *Spheniscus magellanicus* (Chordata) em águas brasileiras

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Abstract

Three new sequences of Mitochondrial cytochrome c-oxidase subunit 2 (mtDNA *cox*-2) from *C. pelagicum* parasite of *Spheniscus magellanicus*, the Magelanicus penguin, were determined from Brazilian waters. The sequences presented 99 and 98% of similarity with *C. pelagicum* sequences from Argentina, deposited on GenBank for the same genetic region and with a strong statistical support inferred from the phylogenetic tree. The morphological and ultrastructural studies that were carried out confirmed the genetic analysis.

Keywords: Anisakidae, penguin, Rio de Janeiro.

Resumo

Foram determinadas três novas sequências da região do Citocromo c-oxidase da subunidade II do DNA mitocondrial (*cox*-2 mtDNA) de *Contracaecum pelagicum*, parasito de *Spheniscus magellanicus*, pinguim Magalhães, de águas brasileiras. As sequências apresentaram 99 e 98% de similaridade com sequências de *C. pelagicum* da Argentina depositadas no GenBank da mesma região genética com forte suporte estatístico inferido pela arvore filogenética. Estudos morfológicos e ultraestruturais realizados confirmaram a identidade genética.

Palavras-chave: Anisakidae, pinguim, Rio de Janeiro.

Introduction

Adult *Contracaecum pelagicum,* Johnston and Mawson, 1942, is an Anisakidae nematode known for parasitizing marine mammals and piscivorous birds. The *Contracaecum* sp. larvae can be found in several invertebrates and in fish that act as intermediate hosts (ANDERSON, 2000).

The species was first described from albatrosses from Australia and, since then the species was reported in South America from *Thalassarche melanophris* (=*Diomedea melanophris* (Temminck, 1828), the South American penguin, *Spheniscus magellanicus* (Forster, 1781), *Spheniscus humboldti* Meyen, 1834 and *Sula leucogaster* Boddaert, 1783 (LENT; FREITAS, 1948; SANTOS, 1984; MANN, 1992; SILVA et al., 2005; GARBIN et al., 2007; GONZÁLEZ-ACUÑA et al., 2008; EDERLI et al., 2009; DIAZ et al., 2010; PRADO et al., 2011; YÁÑEZ et al., 2012; REZENDE et al., 2013; CAMPOS et al., 2013).

Few genetic studies of *C. pelagicum* were performed by Mattiucci et al. (2008) and Garbin et al. (2011, 2013) from the intermediate host, the anchovy *Engraulis anchoita* Hubbs and Marini, 1935, and from the definitive hosts *S. magellanicus* and *Phalacrocorax atriceps* King PP, 1828 from Argentina. However, there are no molecular data of *Contracaecum* species recovered from penguins found in the Brazilian coast.

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Magellan penguins are opportunistic feeders and, in the southern hemisphere during winter time, they may travel along the currents foraging for prey far apart from their original area, the Antarctica and Argentinean coast, reaching the Brazilian southeast and northeast coasts (SERAFINI et al., 2010). In the Brazilian coast, the Magellan penguins are commonly found ill or dead, stranded on the coast (GARCÍA BORBOROGLU et al., 2010). This paper deals with the morphological study and Mitochondrial cytochrome c-oxidase subunit 2 (mtDNA *cox*-2) sequence analysis of *C. pelagicum,* obtained from penguins found in Rio de Janeiro, Brazil.

Materials and Methods

The gastrointestinal tract of two Magelanic penguins was donated by Riozoo foundation at Rio de Janeiro, in December of 2012 and transported in 70% alcohol to the laboratory. One penguin found stranded on the beach was transported to the Laboratory of Parasitology of the Universidade Federal Rural do Rio de Janeiro for examination. A total of 12 adult nematodes (three males and nine females) were collected in the intestine of the penguins, and five were cut into three pieces: the anterior and posterior parts were mounted in glycerine jelly for morphological identification, and the middle parts were prepared for genetic studies. Measurements are given in micrometers (μm) unless otherwise stated, and the means are stated in parentheses. Specimens are deposited in the Helminthological collection of the Instituto Oswaldo Cruz (CHIOC), Rio de Janeiro, Brazil (n. 35913).

Some specimens were prepared for scanning electron microscopy by dehydrating through a graded ethanol series. The specimens were then transferred to 50:50 100% ethanol: hexamethyldisilazane, followed by a change of 100% hexamethyldisilazane and air dried overnight. Mounted specimens were sputter−coated with gold and examined using a Jeol JSM-6791F microscope at an accelerating voltage of 15kV.

Genomic DNA was extracted using a ChargeSwitch gDNA Mini Tissue Kit (Invitrogen, Carlsbad, CA, USA) and in order to amplify gene fragments of *Contracaecum*; the primers 211F/210R for mtDNA *cox*-2 (NADLER; HUDSPETH, 2000) were used in PCR reactions following Borges et al. (2012). Amplified PCR products were purified with Wizard® SV gel and PCR clean up system kit (Promega, Madison, USA), and sequenced using the same primer set. DNA cycle-sequencing reactions were performed using BigDye v.3.1 chemistry (Applied Biosystems, Foster City, CA, USA) in the ABI Prism 3100 sequence analyzer. Sequences were edited in DNASTAR SeqMan (DNASTAR, Inc., Madison, WI) and compared for similarities with sequences from GenBank, using BLAST 2.0 ("Basic Local Alignment Search Tool") (ALTSCHUL et al., 1990) (Table 1). Alignments were performed by CLUSTAL W algorithm (THOMPSON et al., 1994), and the probability of substitutions plus phylogenetic trees were inferred by using the MEGA 5.0 software (TAMURA et al., 2011). The Hasegawa-Kishino-Yano model (HKY) was selected using the jModelTest program (POSADA, 2008), and the Maximum Likelihood method was used to construct trees (FELSENSTEIN, 1981) that were resampled by 1000 bootstrap replicates.

Results

Contracaecum pelagicum was identified based on measurements and morphological observations by light and scanning electron microscopy, in addition to a genetic analysis.

Measurements of males, based on two specimens. Body 40-45 mm long, 0.97-1.16 wide. Anterior region with one dorsal and two ventrolateral lips. Interlabia present. Nerve ring 0.49-0.50 and deirids 0.92-1.00 from anterior end. Oesophagus 2.43-3.82. Ventricular appendix 0.85. Intestinal cecum 1.90-2.69. Right spicule 4.10-4.33 and left 4.00-4.13. Precloacal papillae 23-25 and post-cloacal papillae 7. Cloaca/end of body 0.20-0.24.

Measurements of females, based on four specimens. Body 46-62 (58) mm long and width 1.20-1.50 (1.31). Anterior region with one dorsal and two ventrolateral lips. Interlabia present. Nerve ring 0.48-0.70 (0.60) and deirids 0.71-1.14 (0.91). Oesophagus 3.40-4.15 (4.11). Ventricular appendix 0-70-0.79 (0.74). Intestinal cecum 2.70-3.05 (2.88). Cloaca/end of body 0.45-0.47 (0.43). Vulva 10.80-15.85 (13.32). Egg 0.07 × 0.07. The ultrastructure shows a cephalic collar with cuticle edges directed forward, three long lips (two ventrolateral and one dorsal) intercalated by three triangular interlabia (Figures 1a, b). The morphological analysis confirmed the identification of *C. pelagicum*.

Five samples were used for genetic studies, but only three sequences suitable for analysis were obtained and deposited on

Table 1. List of species from Genbank, used for comparison in phylogenetic analysis and alignments.

Species	GenBank accession number	Reference
Contracaecum pelagicum	EF122210	Mattiucci et al. 2008 Syst. Parasitol. 69 (2): 101-121
Contracaecum pelagicum	EF535568	Mattiucci et al. 2008 Syst. Parasitol. 69 (2): 101-121
Contracaecum pelagicum	EF535569	Mattiucci et al. 2008 Syst. Parasitol. 69 (2): 101-121
Contracaecum multipapillatum	AF179910	Nadler and Hudspeth 2000 J. Parasitol. 86 (2): 380-393
Contracaecum micropapillatum	EU852350	Mattiucci et al. 2010 Syst. Parasitol. 75 (3): 207-224
Contracaecum microcepahulum	EF513519	Mattiucci et al. 2008 Syst. Parasitol. 69 (2): 101-121
Contracaecum chubutensis	HQ328504	Garbin et al. 2011 J. Parasitol. 97 (3): 476-492
Contracaecum septentrionale	EF513513	Mattiucci et al. 2008 Syst. Parasitol. 69 (2): 101-121
Contracaecum rudolphii	EF122201	Mattiucci et al. 2008 Syst. Parasitol. 69 (2): 101-121
Contracaecum rudolphii	EF513502	Mattiucci et al. 2008 Syst. Parasitol. 69 (2): 101-121
Sulcascaris sulcata	HQ328505	Garbin et al. 2011 J. Parasitol. 97 (3): 476-492

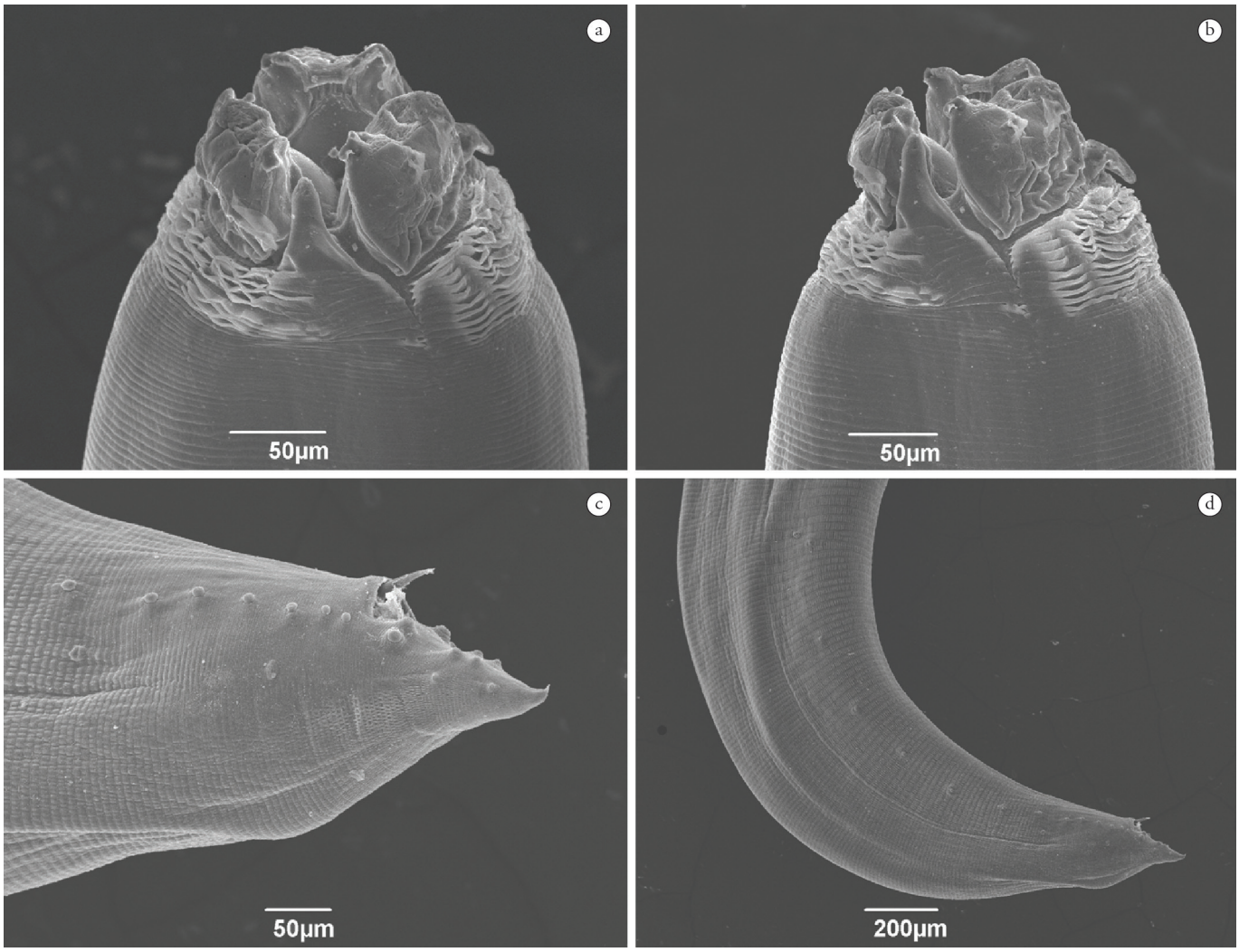

Figure 1. Scanning electron microscopy of *Contracaecum pelagicum* from *Spheniscus magellanicus*. a, b - Detail of anterior end, with conspicuous head collar, lips and interlabia. c - Male caudal end with detail of the pre and post-cloacal papillae, cloaca and spicule tip. d - General view of posterior end of male with distribution of pre and post-cloacal papillae.

GenBank under the accession numbers KC435447, KC435448 and KC435449. These sequences, one with 472 and two with 536 bp, aligned with *C. pelagicum* reference sequence, accession number EF535568 from GenBank with 99% of similarity; with *C. pelagicum* accession numbers EF122210 and EF535569, presented 98% of similarity. The sequences were AT rich with frequencies of 71.1%, 58.8% and 63.8% in first, second and third positions of codons, respectively. Only transitions were observed in relation to other *C. pelagicum*, with a greater rate of T/C substitutions (Table 2). The majority of substitutions occurred in the first position of codons in potentially silent sites. The phylogenetic tree, constructed with the sequences from these studies and the *Contracaecum* reference sequences from birds, showed strong statistical support for the *C. pelagicum* branch (Figure 2).

Table 2. Maximum composite likelihood estimate of the pattern of nucleotide substitution among *Contracaecum pelagicum*. The table shows the probability of substitution from one base (row) to another (column). Transitional substitutions are shown in bold and transversional substitutions are shown in italics.

%	A	T	C	G
A	-	*0.05*	*0.02*	**16.21**
T	*0.03*	-	**15.49**	*0.03*
C	*0.03*	**51.9**	-	*0.03*
G	**16.15**	*0.05*	*0.02*	-

% - percentage.

Discussion

The measurements are in accordance with the data from literature, although the parasites found are much larger than previous descriptions from the same host (SANTOS, 1984; GARBIN et al., 2007). Other morphological features such as number of papillae, length and shape of spicules in males, were similar to the ones previously described for *C. pelagicum* (LENT; FREITAS, 1948; SANTOS, 1984; FAGERHOLM et al., 1996; GARBIN et al., 2007), thus confirming the morphological diagnosis.

The mtDNA *cox*-2 fragments showed little variation among sites of *C. pelagicum*. The intraspecific variability of 1-2% found is in accordance with literature data for Nematodes

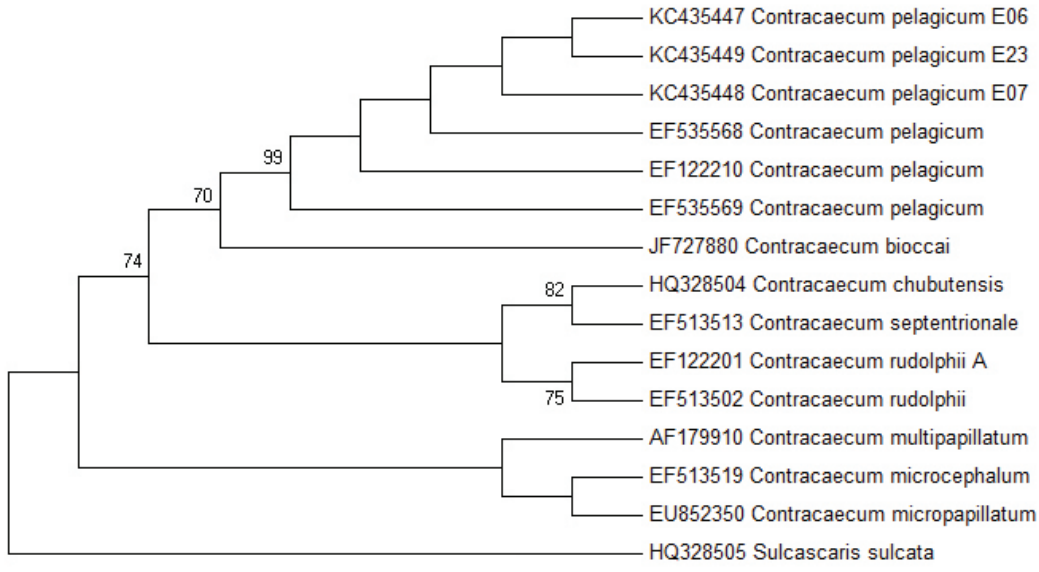

Figure 2. Maximum likelihood reconstruction among Contracaecum pelagicum sequences obtained in this study (E06, E23, E07) and sequences of Contracaecum species from fish eating birds obtained on GenBank, with tree inferred from mtDNA cox-2 data. The numbers on the tree branches represent the percentage of bootstrap resampling. Sulcascaris sulcata was used as an out group.

(THOMAS; WILSON, 1991; GARBIN et al., 2013). In the last 20 years, genetic analysis has been used as an important tool in the identification of sibling species, as well as larval stages of *Contracaecum* (NASCETTI et al., 1993; ORECCHIA et al., 1994; MATTIUCCI et al., 2002; LI et al., 2005; GARBIN et al., 2013). The small intraspecific variation and the greater interspecific variation found in this study, show how the mtDNA *cox*- 2 region can be a valuable tool for the identification of species in the genus.

The AT rich composition found has already been reported in literature for Nematode mtDNA *cox*-2 region (THOMAS; WILSON, 1991; NADLER; HUDSPETH, 2000; JEX et al., 2008). Mattiucci et al. (2008) and Garbin et al. (2013) found values of AT bases frequencies that were very similar to the ones found in this *C. pelagicum* study.

The conducted phylogenetic analysis showed a close relationship between *C. pelagicum* specimens and *C. bioccai,* which formed a strong statistically supported branch. This close relationship between the two species has been already reported in other studies (GARBIN et al., 2013).

Although there were already *C. pelagicum* sequences deposited on GenBank, the addition of new data is important to strengthen the reliability of GenBank as a tool for rapid species' identification, and a database for genetic studies. The new sequences deposited on GenBank provide new data for further studies of these parasites that may well be present in marine fishes along the Brazilian coast.

Conclusion

This is the first genetic characterization of *Contracaecum pelagicum* from *Spheniscus magellanicus* penguins in Brazil.

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